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14. ABSTRACT

**Objectives The ecological success of invasive brown treesnakes (Boiga irregularis) on Guam is related to a lack of natural population controls in the non-native environment. While certain techniques have successfully reduced the number of snakes entering ports and other critical infrastructure, limited research has explored the prospects for biological control as an additional management tool. We proposed to use the phylogenetic relationships of B. irregularis populations spanning the native species? range as an evolutionary map for targeting native populations to survey for parasites. Our rationale was that once parasites could be characterized in native populations representing a range of phylogenetic distances to the Guam snakes, it would be possible to develop a candidate list of parasites for experimental studies on biocontrol efficacy. Our objectives address these endeavors by (1) genetically identifying the source population(s) for introduced Boiga on Guam, (2) providing preliminary phylogenetic data upon which to base decisions for parasite prospecting, (3) testing the longstanding presumption that Guam B. irregularis are parasite free, (4) assessing whether parasites from the source population persist on Guam and (5) providing an initial characterization of metazoan and protozoan parasites in B. irregularis populations within its native range. Technical approach We used DNA sequence data from five genetic markers and Bayesian modeling to infer a phylogeny for B. irregularis populations occurring over much of the native range. We used these same data to compare indices of genetic diversity in the native and introduced range, as low genetic variation on Guam may indicate a reduced immunological capacity to respond to infection. We harvested parasites from field-captured snakes at two locations, one representing the putative source of the Guam population and one representing ?mainland? New Guinea, and identified parasites to the lowest taxonomic rank possible based on morphology. We also conducted parasite surveys on Guam to test whether invasive B. irregularis continue to harbor parasites from the source population. Results We recovered a single mtDNA haplotype and limited nuclear genetic variation in 24 treesnakes captured across Guam, suggesting that this population was founded by a small number of individuals from a single source location. DNA sequence data verifies the source in the Admiralty Islands off the north coast of New Guinea. Contrary to previous assertions, Guam snakes do harbor helminth parasites, although prevalence, infection intensity, and species diversity is low. We found that Papua New Guinea populations were heavily infected with a variety of helminth and haemoparasite species, none of which were recovered from treesnakes on Guam. Our results satisfy the proof-of-concept necessary to pursue further lines of investigation on biocontrol. Benefits Eradication of B.**

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## List of Acronyms

mtDNA – mitochondrial DNA
nuDNA – nuclear DNA
PCR – polymerase chain reaction
SVL – snout-to-vent length

**Keywords**: *Boiga irregularis*, Papua New Guinea, Guam, biological control, phylogeography, parasites, haemogregarines

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# Abstract


## Objectives

The ecological success of invasive brown treesnakes (*Boiga irregularis*) on Guam is related to a lack of natural population controls in the non-native environment. While certain techniques have successfully reduced the number of snakes entering ports and other critical infrastructure, limited research has explored the prospects for biological control as an additional management tool. We proposed to use the phylogenetic relationships of *B. irregularis* populations spanning the native species' range as an evolutionary map for targeting native populations to survey for parasites. Our rationale was that once parasites could be characterized in native populations representing a range of phylogenetic distances to the Guam snakes, it would be possible to develop a candidate list of parasites for experimental studies on biocontrol efficacy. Our objectives address these endeavors by (1) genetically identifying the source population(s) for introduced *Boiga* on Guam, (2) providing preliminary phylogenetic data upon which to base decisions for parasite prospecting, (3) testing the longstanding presumption that Guam *B. irregularis* are parasite free, (4) assessing whether parasites from the source population persist on Guam and (5) providing an initial characterization of metazoan and protozoan parasites in *B. irregularis* populations within its native range.

## Technical approach

We used DNA sequence data from five genetic markers and Bayesian modeling to infer a phylogeny for *B. irregularis* populations occurring over much of the native range. We used these same data to compare indices of genetic diversity in the native and introduced range, as low genetic variation on Guam may indicate a reduced immunological capacity to respond to infection. We harvested parasites from field-captured snakes at two locations, one representing the putative source of the Guam population and one representing 'mainland' New Guinea, and identified parasites to the lowest taxonomic rank possible based on morphology. We also conducted parasite surveys on Guam to test whether invasive *B. irregularis* continue to harbor parasites from the source population.

## Results

We recovered a single mtDNA haplotype and limited nuclear genetic variation in 24 treesnakes captured across Guam, suggesting that this population was founded by a small number of individuals from a single source location. DNA sequence data verifies the source in the Admiralty Islands off the north coast of New Guinea. Contrary to previous assertions, Guam snakes do harbor helminth parasites, although prevalence, infection intensity, and species diversity is low. We found that Papua New Guinea populations were heavily infected with a variety of helminth and haemoparasite species, none of which were recovered from treesnakes on Guam. Our results satisfy the proof-of-concept necessary to pursue further lines of investigation on biocontrol.

## Benefits

Eradication of *B. irregularis* on Guam would have tremendous ecosystem, health and economic benefits, and would reduce the risk of further introductions into other non-native areas. Benefits specific to biological control include reduced human intervention and therefore reduced cost to sustain management, given that an effective bio-control agent would self-deploy. Our evolutionary approach to using parasites for treesnake management demonstrates the potential of our work to broaden the scope and efficacy of control tactics already in place on Guam.

# Objectives

Our SEED project evaluates the feasibility and effectiveness of using host phylogenetic data as a search criterion for potential biological agents, and begins the process of characterizing the many parasites and pathogens that infect *Boiga irregularis* in the tropical native range. Specifically, our objectives were to provide baseline data by (1) genetically verifying the source of the invasive population and testing whether its constituents arrived from different places, (2) providing preliminary phylogenetic data upon which to base decisions for parasite prospecting, (3) verifying or refuting the longstanding presumption that Guam *B. irregularis* are parasite free, (4) assessing whether parasites from the source population persist in the invasive population and (5) providing an initial characterization of metazoan and protozoan parasites in native populations. Although a source population has been postulated based on historical military records and morphological similarity, studies to date have not verified the identity of this population using modern genetic techniques, nor has the possibility of multiple introductions from separate locations been ruled out.

Our study design capitalizes on the longstanding notion that the Guam population has been purged of its native parasites (D. Nichols, pers. comm., 1992 in Rodda et al. 1999a), a pattern observed in many invasive taxa following their introduction into foreign habitats (i.e. 'parasite release': Torchin et al. 2003, Marr et al. 2008). After approximately 60 years of persisting on the island, we hypothesize that the newest generation of treesnakes may have increased vulnerability to former co-evolved parasites, and may be immunologically repressed due to the effects of an extremely small founder population (i.e. low immunogenetic diversity, and therefore a reduced capacity to respond to an array of pathogens). This vulnerability may increase as the amount of evolutionary divergence between the invasive and native *B. irregularis* populations grows larger, as immunological responsiveness to certain parasites and pathogens may decrease over time if exposure is limited or absent.

To begin assessing the prospects for biological management, we developed an evolutionary-based approach to examining how native populations might be naturally kept in check by parasites, thereby increasing the probability of identifying a host-specific agent with deleterious effects on the fitness of invasive snakes. The use of DNA sequence data to reconstruct a phylogenetic hypothesis for *B. irregularis* populations throughout the native range provided a straightforward starting point upon which to pursue this endeavor. With a statistically robust phylogenetic reconstruction (or 'tree'), we could then identify the most likely source(s) of the Guam population and test whether it was founded from a single or multiple source locations. We could then work backwards in time, so to speak, using the tree as an evolutionary guide for targeting native populations that differ in their level of relatedness to the Guam population for parasite assays; the rationale for this strategy is that 'phylogenetic latitude' in our choice of study populations would yield a broad spectrum of native pathogens or parasites from which to choose the best possible candidates for biological control. By focusing across populations rather than on the source population alone, we increase the probability of finding candidates that satisfy certain requirements related to transmission, virulence, and degree of host specificity.

In addition to a lack of exploratory research on this form of management, our approach differs from previous efforts by combining host genetic data with assays of natural parasite faunas. This enables the development of a biologically informed framework regarding the best candidates to pursue for experimental studies on virulence and transmissibility. Our intent with this approach is to maximize the chance of finding an effective, host-specific agent while simultaneously minimizing the possibility of creating new environmental problems on Guam.

***Our proposal responds to the SERDP statement of need by pursuing a largely unexplored form of treesnake population control that has the potential to be less labor intensive and more cost effective when compared to current control techniques (i.e. trapping, visual searchers, dog teams, etc.) – we approach the subject using an innovative method that combines information on host genetics and naturally occurring enemies of the snake to arrive at an effective management strategy, and advocate for the use of biological management in conjunction with other control measures. In essence, our goal is to use the evolutionary ecology of B. irregularis as a weapon against the snake itself.*** Our emphasis on biological management is well supported by theoretical models that demonstrate the strong potential of several different types of pathogens or parasites to regulate treesnake abundance on Guam (Dobson and Altizer 2001). This work is timely due to US military base consolidation in East Asia and the western Pacific, which is predicted to significantly increase the frequency, quantity, and range of goods and people transported to and from Guam over the next several years (Erickson and Mikolay 2008, Campbell 2009). In fact, major redeployment of air and naval assets is already under way (Kan and Niksch 2009). Because *B. irregularis* is adept at clandestinely entering ships and aircraft, increased traffic to and from the island will simultaneously increase the likelihood of introductions into other ecologically sensitive areas, including the continental U. S., unless effective methods for controlling snake abundance island-wide are established (Wisniewski 2010). Estimates of the annual economic costs from the damages caused by the invasion of *B. irregularis* to the Hawaiian Islands from Guam range from $.59B - $2.14B (Shwiff et al. 2010).

# Background

Introductions of non-native vertebrates to island ecosystems have caused major declines and shifts in the community structure of endemic birds, reptiles, and native vegetation (e.g. Cruz and Cruz 1987, Dobson 1988, Chapuis et al. 1994). Moreover, invasive vertebrate species often cause great agricultural and economic losses for humans and have been implicated in the spread of infectious diseases to wildlife and domestic animals (Hone 1994, Feldman et al. 1995, Case 1996). The brown treesnake, *Boiga irregularis*, is an arboreal, mildly venomous snake that has reached iconic status as one of the most destructive invasive species of modern times. Native to Indonesia, north-northeastern Australia, New Guinea, and several larger Pacific Islands (Fig. 1), this predator was accidentally introduced to Guam sometime in the late 1940s (Rodda *et al.* 1992) where it has had devastating effects on the ecology and economics of the island. The reasons underlying this snake's success and the need to eradicate or control population density on Guam is discussed in numerous papers spanning more than two decades (e.g. Fritts 1987, Savidge 1987, Conroy 1988, Fritts and McCoid 1991, Rodda et al. 1992, Fritts and Rodda 1998), many of which are summarized in an edited book volume that is largely dedicated to invasive *B. irregularis* (Rodda et al. 1999a). For purposes of this report, we focus on a brief review of the types and efficacy of existing control methods, and continue building a case for using biological management as a way to augment existing control techniques.

The potential for escape or colonization of *B. irregularis* to other non-native locations is a continued threat, as civilian and military traffic leaving Guam is frequent and the density of snakes is high, even in urban and developed areas. Although *B. irregularis* densities on Guam have declined from a peak in the mid-1980s, they are still far beyond those observed in their native range (Savidge 1991, Rodda et al. 1999b). A number of control strategies have either been proposed or implemented, including bait-trapping and removal (Campbell III 1999b), barrier fencing (Perry et al. 1998, Campbell III 1999a), prey base control (Vice and Pitzler 2000), use of chemical agents to attract, repel, or disrupt the reproductive biology of the snakes (Savarie and Bruggers 1999, Greene and Mason 2003), interception of snakes using canine detection (Engeman et al. 1998, Vice and Engeman 2000, Savidge et al. 2011), early detection and rapid response teams (Stanford and Rodda 2007), community outreach (Martin 2007, Hawley 2007), and increased port biosecurity efforts (Kraus and Cravalho 2001). More recent approaches involve baits treated with acetaminophen distributed among bait stations (Savarie et al. 2001; acetaminophen is lethal to treesnakes at proper dosages), or the aerial bombardment of acetaminophen-laced mice using parachute-like devices designed to keep the bait within the forest canopy (Shivik et al. 2002, Savarie et al. 2007). While these approaches have demonstrated some success, none have adequately resolved the problem

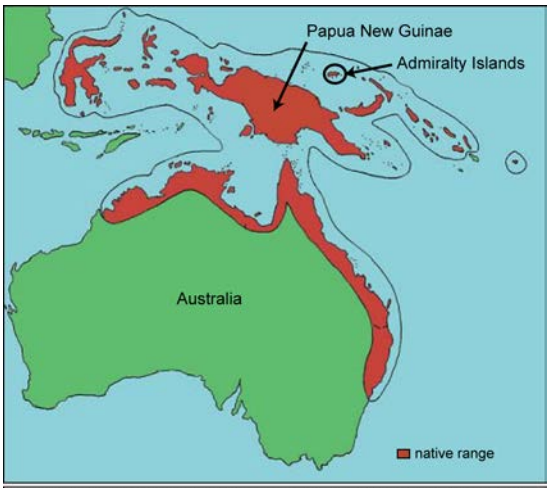


Figure 1. Map displaying the native range of *Boiga irregularis* (modified from www.fort.usgs.gov/resources/education/bts/bioeco/btsnake.asp).

to date. Perhaps most limiting to these techniques is that all of them require continual labor and expense to sustain effectiveness, a factor that is potentially eliminated with a self-disseminating biological agent.

It has long been recognized that parasites may regulate host populations at levels well below the carrying capacity set by resources (e.g. Anderson and May 1978), and because of its cost-effective nature and potentially sustainable regulation of target organisms, biological control is often viewed as an attractive option relative to physical and chemical methods (van den Bosch et al. 1982). Hundreds of biological control programs have targeted insect and weed pests over the past century and have met great success (Waage and Mills 1992, Holt and Hochberg 1997); however only a few examples of successful biological control for vertebrate pests exist (Davies et al. 1976, Fenner and Fantini 1999). Failures are attributed mainly to insufficient host specificity, or the non-specific foraging habits of introduced predators (Hone 1994 , Fenner and Fantini 1999). Indeed, several disastrous cases involving the deployment of vertebrate predators for the control of other pests has cast a dark cloud over the involvement of vertebrates in any aspect of biological control, whether they be the targets of control or the control agents themselves. The fact remains however, that cases involving vertebrates as targets of biological management are few and far between, leaving little evidence by which to judge the general efficacy of this technique in the management of vertebrate pests.

Although few successful examples exist, the concept of deploying a parasite or pathogen to manage invasive vertebrates is widely advocated in the literature. Examples include the chytrid fungus (*Batrachochytrium dendrobatidis*) for invasive frogs (Beard and O'Neill 2005), a liver nematode (*Capilaria hepatica*) for the house mouse (*Mus musculus*) (McCallum and Singleton 1989), swine fever for the feral pigs (*Sus scrofa*) (Nettles et al. 1989), *Salmonella* spp. for rodents (Singleton 1994 ) and numerous others. 'Real world' examples of successful biological management of vertebrate pests involve the use of myomatosis, myxoma virus and rabbit hemorrhagic disease (RHD) to control feral rabbit populations around the world (Fenner and Ratcliffe 1965, Cooke and Fenner 2002, Forrester et al. 2006), and feline panleucopaenia virus against invasive cats on the small sub-Antarctic Marion Island (van Rensburg et al. 1987). In fact, more than 50 years post-release, myxoma virus is still lethal to a large proportion of rabbits born in Australia each year, at zero cost or physical expenditure (Kerr et al. 2003).

A number of key factors suggest that the treesnake problem on Guam is amenable to a similar approach (Dobson and Altizer 2001), particularly if a biological agent is used in conjunction with other conventional techniques. First, the snake population density is high, which can enhance and accelerate disease transmission. The fact that these snakes are virtually unlimited in terms of the places they occur on Guam could translate to a rapid spread of a pathogen across the entire island. Second, *B. irregularis* is one of only two snake species on the Guam, the other of which is a burrowing blind snake *Ramphotyphlops braminus* (possibly introduced as well), and none of the other nearby islands have native snakes. Thus, with a host- or even snake-specific agent, the probability of cross-transmission is low due to limited ecological overlap with *R. braminus*. Third, there is little concern that a snake biocontrol could escape outside of the targeted area because Guam is a relatively remote island (although snake escape would remain an issue). Finally, Guam is small enough (541.3 $km^2$) that the agent could deploy fairly easily and quickly, especially

because the treesnake population density is so high. Despite these encouraging factors, identifying a suitable agent is no easy task, and careful laboratory and field-testing must be performed to ensure that introduced pathogens or parasites do not affect non-targeted hosts.

Little is known about *B. irregularis* parasites because few parasitological surveys have been done in the native range (but see Whittier et al. 2003; Jakes et al. 2003). Anecdotal evidence from nearly two decades ago suggests that *B. irregularis* on Guam have been released of their former co-evolved parasites (Rodda et al. 1999a), but whether this assertion was or is true remains unclear. Such 'enemy release' is not uncommon in invasive, non-native taxa (Torchin et al. 2003, Marr et al. 2008), and could work to the advantage of biological management in *B. irregularis* if the loss of former parasites has resulted in a reduced capacity to respond to infection. The idea of using a biological agent is not new, and experimental studies involving cutaneous mites and paramyxoviruses have been pursued previously (Fritts and Scott 1985, D. Nichols in litt. 1992, 1996, 1999, 2000). Paramyxoviruses cause flu-like symptoms in captive snakes, and experimental evidence suggests that certain strains may be effective at killing *B. irregularis*. However, nothing is known about the virulence of paramyxovirus in wild *B. irregularis* populations and it is unclear whether strains showing purported effects in captivity would transmit in nature. More recent studies have detected a virulent novel strain of orthoreovirus in rough green snakes (*Opheodrys aestivus*) that might also be an option for experimenting with *B. irregularis* (Landolfi et al. 2010).

Other candidates that attracted early attention for controlling *B. irregularis* include haemogregarine blood parasites, which are the dominant haemoparasites in snakes, turtles and crocodilians (Telford Jr. 1984). Haemogregarines are known from *B. irregularis* populations in New Guinea and Australia (Mackerras 1961, Ewers 1968), but the taxonomic identities of these species and their degree of host-specificity remain unclear. Numerous haemogregarine species use haematophagous arthropods (mosquitoes, sand flies, acarinids, and mites) to complete the life cycle, and many of these arthropods are known to feed on snakes (Ball et al. 1967, Telford Jr. 1984, Wozniak and Telford Jr. 1991, Telford Jr. 1999). Furthermore, representatives of these types of intermediate hosts naturally occur on Guam.

To add to the demographic and biological factors that may promote the success of biological management for treesnakes, Dobson and Altizer (2001) developed mathematical models that integrated aspects of the population biology of *B. irregularis* and assessed the likelihood of different types of parasites and pathogens to control the invasive population. They also conducted a literature search of known pathogens of snakes, including *B. irregularis*, and other reptiles to identify a suite of potential biocontrol agents and described their transmission, virulence, and host specificity (28 parasites and pathogens examined, with 10 showing strong potential for success in controlling invasive *B. irregularis*). Their findings suggest that transmission mode will be a key determinant of the rate of spread, post-invasion dynamics, and the ability to regulate treesnake densities on Guam. Specifically, they suggest that parasite species with intermediate virulence will provide the best long-term control, and sterilizing STDs (or those with small effects on mortality) could potentially lead to eradication. Release strategies, disease spread, and possible host-parasite co-evolutionary outcomes were also examined, in addition to gaps in

critical knowledge that would need to be filled before the initiation of any biological control program.

***The question of how and where to prospect for additional parasite candidates has no easy answer, but searching within the framework of the evolutionary history of B. irregularis and its natural parasites may provide a means for reducing the potential risk associated with the introduction of such an agent on to Guam***. *Boiga irregularis* has a fairly limited distribution (Fig. 1) and the geographic origin(s) of the original invaders have never been definitively identified using genetic techniques. Understanding how *B. irregularis* on Guam are placed within the phylogeography of the species will help pinpoint the source population(s) and its associated parasites, and could aid in identifying parasite species that might perform more effectively as a biological agent due to the recent phylogenetic history shared between Guam and source populations. If parasite faunas differ across native *B. irregularis* populations as expected, effective use of the phylogeny as an index (or blueprint) for targeting native populations that span a range of evolutionary relatedness can be realized, thereby providing opportunities to survey a wide taxonomic breadth of parasites and pathogens that have co-evolved with the species but are absent on Guam. This in turn should increase our chances of identifying suitable biocontrol candidates for experimental work. Fortunately, the process of obtaining material for phylogeographic analysis and parasitic surveys are compatible, and the Dobson and Altizer (2001) study provides an excellent foundation for narrowing the search for candidate parasites or pathogens.

We are aware of only one study, an 'as-of-yet' unpublished honor's thesis that has attempted to identify the genetic/geographic origin(s) of *B. irregularis* on Guam; however, the limited amount of data presented in this study failed to provide definitive evidence of a source population and prospective biological controls were not investigated. We expand on this work in a fourfold manner; first, by increasing the geographic sampling of snakes from throughout the native range of the species; second, by increasing the number of genes and the amount of sequence data used to generate a statistically supported phylogeographic inference; third, by providing a more up-to-date and comprehensive screening for possible parasites in *B. irregularis* and other non-targeted reptilian hosts from Guam; and fourth, by comparing any parasites recovered from Guam snakes with the parasites of conspecifics collected from different native populations with varying degrees of evolutionary relatedness, including the source population.

# Materials and methods

## Field surveys and tissue collection

We focused our field sampling at six locations on Guam and two locations in the native range in Papua New Guinea (Fig. 2). For Guam, we spread our efforts over the entire island to maximize the probability of capturing all of the genetic variation that exists within the invasive population. Study sites in Papua New Guinea included the Admiralty Archipelago in the Manus Province, specifically Manus, Los Negros, and Pityilu Islands, and the Kamiali Wildlife Reserve in the Morobe Province. We selected these sites for our initial work in the native-range for two main reasons: (1) the source population for Guam *B. irregularis* is suspected of being on the Admiralty Islands (Rodda et al. 1992), so studying treesnakes from these islands allowed us to genetically verify this hypothesis and to sample parasites from the putative source population; (2) previous field surveys by professional colleagues indicated that *B. irregularis* were fairly common in secondary forest in Kamiali, and could potentially provide a suitable number of snakes for parasite prospecting from a 'mainland' Papua New Guinea source.

We captured as many snakes as possible by hand during night surveys and augmented hand captures on Guam using mouse-baited traps. Bait traps were not used in New Guinea due to the potential for capturing numerous non-targeted snake species and difficulties in transporting and maintaining fresh bait for the duration of the fieldwork. Individuals were weighed and measured following a light intracoelomic injection of tricaine methanesulfonate (enough to sedate), followed by collection of blood samples on to several media as described below. Once we obtained adequate blood samples, we euthanized the snakes using an intracardial injection of tricaine methanesulfonate following the methods of Conroy et al. (2009). We immediately excised a small piece of liver tissue in RNAlater® stabilization buffer and a second subset in 95% ethanol for DNA analysis; whole snake specimens were pickled in formalin for a minimum of 48 hours, and then placed in to 70% ethanol for long-term storage.

For the molecular study, we obtained additional *B. irregularis* tissues from other areas in the native range from professional colleagues and or museum collections. We also included samples from eight other *Boiga* species to use as outgroups for the phylogenetic analysis. These included *B. nigriceps*, *B. dendrophila*, *B. cynodon*, *B. multomaculata*, *B. kraepilini*, *Toxicodryas* (formerly *Boiga*) *blandingii*, *T. pulverulenta*, and *B. drapiezii.*

## Collection and identification of parasites

Using standardized protocols (Gardner et al. 2012), we harvested parasites from all snakes the morning following capture – weight, snout-vent length, sex, reproductive condition, fat stores, and evident pathology were noted for association with infection levels. We noted any injuries or other external abnormalities, alertness (responded defensively to handling versus lethargic), and stage of skin shedding (ecdysis). Prior to euthanization, we collected blood samples using heparinized capillary tubes and prepared a minimum of two thin blood smears per individual. Smears were prepared on standard microscope slides and fixed in 100% methanol. We also blotted a small amount of blood onto FTA® cards for later hemoparasite DNA extraction. A subsample of smears were subjected to Giemsa staining and examined for parasites under light microscopy at different magnifications and for

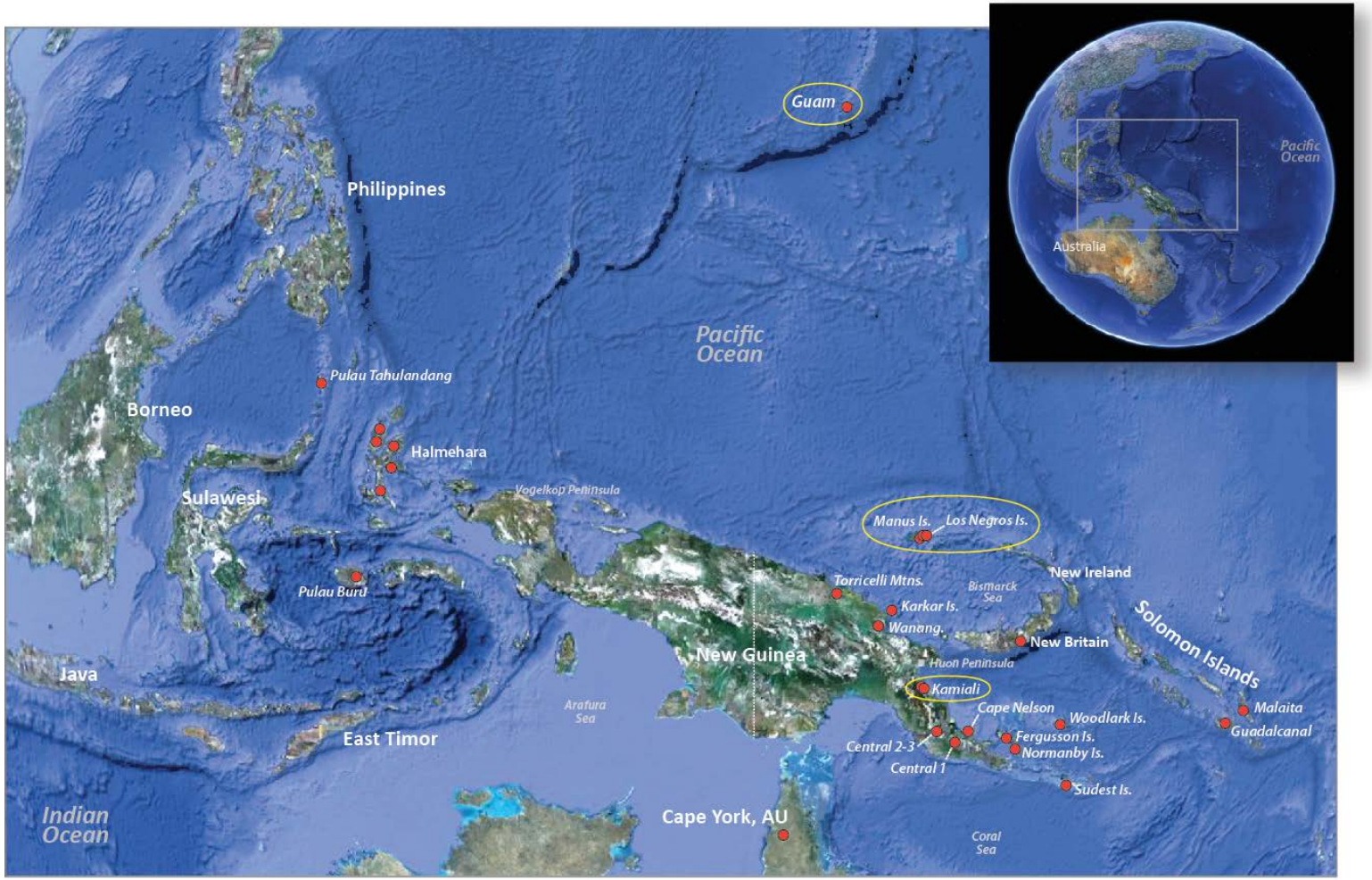



**Figure 2.** Sampling locations for this study. Inset global map details the focal area. Red bullets indicate locations sampled for genetics; yellow ovals indicate areas sampled for parasites. The dashed line through the center of New Guinea demarcates Indonesian Provinces to the west (Papua and West Papua) and the country Papua New Guinea to the east.

different time increments, following the protocols of (Schall 1996). A series of non-stained samples were also preserved and archived for later antibody tests for viral agents. Blood parasites were isolated and identified in the laboratory by Dr. Sam R. Telford Jr. (Florida Museum of Natural History and University of Florida, Gainsville).

Immediately following euthanization, we conducted a thorough visual screening for ectoparasites and placed any recovered specimens in 70% ethanol. We then opened the body cavity by making a longitudinal ventral incision from throat to vent, and dissected the esophagus, stomach, intestines, lungs, liver, heart, kidney, and urinary bladder. Each organ was placed into hot saline solution in separate Petri dishes and examined under a dissection microscope in the field. Nematodes were temporarily stored in 70% ethanol; cestodes in trematodes were temporarily stored in 70% ethanol or placed directly in to neutral buffered 10% formalin. Helminth parasite identifications were performed in the laboratory by Dr. Charles R. Bursey (Penn State University, Shenango) according to the following protocols: nematodes were placed into glycerol, allowed to clear, and examined under a light microscope; cestodes were regressively stained in Delafield's hematoxylin, dehydrated using a graded series of alcohol, cleared in xylene, and mounted in balsam for examination; pentastomids were dehydrated in graded alcohol and cleared in cedarwood oil for examination.

To begin evaluating parasite host specificity in the different sampling locations, we opportunistically sampled blood and harvested helminths from additional snake species that co-occur with *B. irregularis* in Papua New Guinea. We also screened for parasites in the only other snake species that occurs on Guam, the blind snake *Ramphotyphlops braminus*, which may also be non-native.

**Molecular data collection**

The main goal of the molecular part of this study was to pinpoint the source (or potential sources) of the invasive population. This goal could be easily met by using DNA sequence data from a relatively small number of mitochondrial and nuclear genes to examine the distribution of evolutionary lineages within *B. irregularis* across its native range. With this type of phylogeographic data, we can then focus future efforts on quantifying genetic diversity in populations at small, intermediate, and large evolutionary distances to the Guam population. Such analyses require greater numbers of individuals and molecular markers than explored here; however, decisions on which populations and locations to target for additional genetic comparisons would not be possible without the types of preliminary data and analyses presented in this study.

We extracted DNA from liver tissue using a Qiagen DNeasy blood and tissue kit. Archival DNA samples for all specimens are currently held in ultracold frozen storage (-80ºC) at the USGS San Diego Field Station. We used sequence data from both mitochondrial and nuclear genes to estimate a phylogenetic tree for *B. irregularis* and to calculate various genetic diversity indices for the sampled populations. For mitochondrial DNA (mtDNA), we sequenced the NADH dehydrogenase subunit 4 gene (ND4) and portions of the upstream flanking tRNA genes, as these markers have proved useful in other phylogeographic studies in snakes (Williams et al. 2008; Wüster et al. 2004). For nuclear DNA (nuDNA), we screened six genes for sequence variability across the species' range, and identified four that provided suitable variation for distinguishing *B. irregularis* populations. These

included an intron of the TATA-box binding protein (*Bi*TBP), the recombination activation gene subunit 1 (RAG-1), the proto-oncogene *Cmos*, and the myosin heavy chain type II (MyHC-2). All genes were amplified using standard polymerase chain reaction (PCR) with the following thermocycling parameters: 95ºC-3:00 (initial denaturation); 95ºC-30s, 60ºC-30s, 72ºC-45 for 30 cycles; 72ºC-10:00 min extension. The annealing temperatures varied between 58-62ºC depending on the primer set. We visualized the PCR products on 1% agarose gels prior to sequencing on an ABI 3730 automated DNA sequencer using Big Dye v3.1 chemistry.

## Genetic diversity analyses

Although our study was not designed to conduct rigorous statistical comparisons of genetic diversity across the range, we collected sufficient data to provide at least some insight on patterns of regional variation. The overall genetic diversity within a population can be used as a proxy for how well its constituents can potentially cope with pathogens or disease (assuming that variation from five neutral genetic markers is representative of the genome as a whole, and therefore of genes involved with immune function). Consequently, we compared the diversity of individuals from different geographic areas for both mtDNA and nuDNA using a variety of diversity statistics, including number of haplotypes/alleles, haplotype/allelic diversity ($H_d$), nucleotide diversity ($\pi$), and number of polymorphic sites ($s$). All diversity indices were estimated using the program DNASP 5.0 (Librado & Rozas 2009). We restricted these analyses only to areas where we had sequence data for more than three individuals, and in some cases we pooled data from different locations if there was contiguous habitat separating collection sites. If the Guam population was founded by a small number of individuals from a single location, we would expect Guam diversity estimates to be markedly lower than any other location in the native range. Also, we would expect small-island populations in general to have less diversity than large-island populations or populations on 'mainland' Papua New Guinea.

To test for the possible presence of multiple mtDNA haplotypes on Guam, we sequenced 24 snakes from the eight sampling sites for ND4 in one direction (~800 bp). We predicted that this sample size and amount of sequence, as well as the geographic coverage of the island, was sufficient to capture all of the mtDNA diversity that currently exists on Guam. If more than one haplotype was recovered from this initial batch of samples, our approach was to continue sequencing until no further unique haplotypes were detected.

## Resolving allelic phases for heterozygous individuals

Because all nuclear genes in diploid organisms are bi-allelic, it is possible for individuals to carry two unique alleles at any given locus (i.e. heterozygote). These polymorphisms come in two forms, one in which alternative alleles differ in sequence length and one in which homologous sites within the sequence display different nucleotides. Direct sequencing of such templates produces two superimposed allelic traces with either a phase shift (in the case of length variation) or two peaks overlapping at a single nucleotide site, making it difficult or impossible to reconcile the sequence of the true allele.

In cases where we detected heterozygotes through an initial round of sequencing, we resolved the allele phases using two approaches, one using computer optimization and

the other by cloning the PCR products. We relied on computer optimizations for nearly all cases and used cloning mainly to generate a few reference sequences to verify that the programs were accurately reconstructing the alleles. We describe the different computer optimization methods below. For cloning, PCR products from reactions containing known bi-allelic templates (as determined from an initial round of sequencing) were cloned into a pGEM® T vector (Promega, Madison, WI, USA), and recombinant DNA was transformed into TOP-10 *Escherichia coli* cells (Invitrogen). We grew the *E. coli* cells on Luria agar plates for 18-20 hrs at 37°C, and used blue/white screening to randomly choose 8 positive clones to amplify using M13 primers. PCR products were then visualized, purified and sequenced as described above. In all cases, we were able to resolve the two alleles of a given nuclear locus.

For heterozygotes in which we detected length polymorphisms, we used the software INDELLIGENT (Dmitriev and Rakitov 2008) to estimate the pair of maximally similar allelic strings that could be superimposed to produce the observed pattern of peaks. This method yields accurate reconstructions when the allelic sequences are highly similar, the nucleotide insertion or deletion is small relative to the length of the analyzed fragment, and multiple insertions or deletions, if present, are well spaced. Our samples met each of these criteria, and in no cases were the number of resolved positions for a given sequence any less than 96% (as determined by the output from INDELLIGENT). We tested the accuracy of the inferred alleles by phasing templates in both the forward and reverse directions, and creating consensus strands to ensure that the same nucleotide calls were being made in both directions.

For individuals in which we recovered site-specific polymorphisms, we used a Bayesian predictive method implemented in the software package PHASE to resolve phase states (Stephens et al. 2001; Stephens and Donnelly 2003). If the phase of a polymorphic site could not be inferred with a posterior probability above 0.90, that site was coded using IUPAC nucleotide ambiguity codes and therefore treated as missing data in the phylogenetic analyses.

## Phylogenetic analysis

The objectives of this analysis were to genetically identify a source population for Guam *Boiga*, to test whether snakes may have been introduced from multiple locations, and to create a phylogenetic 'blueprint' for making decisions about where to conduct parasite prospecting. If treesnakes originated from multiple sources, we expected to find different mtDNA haplotypes on Guam that match those of source populations from different locations. It was also possible that a single source population could contain mtDNA polymorphism, in which case the initial propagules to arrive on Guam could have harbored different mtDNA variants (although the genetic divergence between them would be expected to be low). In either case, our sampling was sufficient to detect either scenario, as well as deeper phylogeographic splits within the species as detected by the sorting of alleles from the different nuclear genes among the different geographic regions.

We included sequence data from all outgroup species and 65 *B. irregularis* from different locations in the native and introduced range. For Guam, we used single individuals collected from eight sampling sites widely spaced across the island as representatives for the phylogeographic analysis (but see Genetic Diversity section below). This level of

sampling on Guam proved sufficient for detecting the source population, based on initial mtDNA diversity estimates obtained from 24 snakes (see Results and Discussion section under 'Genetic diversity analyses). We sequenced all hand-captured snakes from Kamiali and the Admiralties given that we expected a substantially greater amount of genetic diversity in these areas. Outgroup species were represented by single individuals, with the exception of *B. drapiezii* ($n$ = 2).

To perform phylogenetic analyses, we first identified the most appropriate models of nucleotide evolution using the Bayesian Information Criterion (BIC) as implemented in jMODELTEST 2.3 (Posada 2008). We selected models for each of the three codon positions in the ND4 gene, the combined tRNA genes (tRNAs were combined due to their short length), and for each nuclear gene separately, and performed all combined-gene analyses by assigning separate models to the respective gene partitions. For sequences in which we recovered phylogenetically informative insertions or deletions, we coded these sites as binary characters and included these data in our multi-gene analyses, given that the tree inference software used in this study would otherwise treat gaps as missing data. We modeled these changes according to a stochastic Dollo transition model, in which gains in blocks of sequence were considered unlikely compared to losses.

We conducted an initial series of phylogenetic analyses using Bayesian methods in BEAST v1.6.1 (Heled & Drummond 2010). The datasets in this series were as follows: mtDNA only, the four nuclear genes plus a matrix encoding insertions/deletions, and a full concatenation of all datasets (mtDNA + nuDNA). We assumed a Yule speciation process tree prior and ran two independent tree searches of 30 million generations each, retaining every 1000$^{th}$ sample from the posterior distribution of the model parameter log files and tree files. We deviated slightly from this scheme in the full-concatenated analysis by combining data from two independent searches of 20 million generations each ($n$ = 40 million generations retaining every 1000$^{th}$ sample; Yule process tree prior). We evaluated whether model parameters had reached stationarity and converged on similar values between independent runs using the programs TRACER v1.4.1 (Rambaut and Drummond 2007) and AWTY (Nylander *et al*. 2008). Branch support for the different tree topologies was evaluated by the posterior probabilities ($Pp$) of the inferred relationships, where $Pp_s \geq 0.95$ were considered strongly supported.

We further analyzed the data using the *BEAST option in BEAST v1.6.1 (Heled & Drummond 2010), given the ability of this method to jointly infer a summary tree from different gene trees (i.e. genealogies) sampled from multiple individuals. Conceptually, a *BEAST analysis amounts to embedding separate genealogies inside the summary tree following the coalescent process backwards in time, starting from the tree tips (Rannala and Yang 2003). The model assumes that gene flow does *not* occur among the groups of interest (i.e. the tips of the tree), regardless of whether they are taxonomic or geographic entities, and therefore any incongruence between the genealogies and the summary tree is attributed to the retention of ancestral polymorphism. This presumption is appropriate for inter-island populations of the same snake species that does not naturally disperse over water, and therefore does not exchange genes among islands. We considered the *BEAST approach as the best way to exploit all information in the mtDNA and nuDNA datasets in a single analysis, given the ability of *BEAST to account for differences in the time to coalescence between haploid (mitochondrial) and diploid (nuclear) markers. This can

improve the accuracy of tree reconstruction compared to the more traditional approaches that do not account for the coalescent process, assuming that the condition of no gene flow among lineages is met. The *BEAST approach is especially relevant to cases involving recent divergence events, where the retention of ancestral polymorphisms among lineages is more likely to mislead phylogenetic reconstruction.

Because we were primarily interested in testing whether the source population for Guam snakes resides in the Admiralty Islands, we designated groups of individuals from different geographic areas as 'species' for the *BEAST analysis (given that the taxonomic rank of species has no bearing on how *BEAST reconstructs the tree, other than being a user-defined group that fits the assumptions of the coalescent model). We implemented a Yule tree prior and partition-specific substitution models; however, due to difficulty in achieving convergence for these multi-gene parameter-rich models, we did not run codon-partitioned models for the ND4 gene and gaps were treated as missing data. Instead, a single best-fit model was determined for all ND4 sites combined. This had a negligible effect on our results, as the codon-partitioned model for ND4 gave the same topology as a non-partitioned model (in the single gene analysis) and very few informative gaps existed within *B. irregularis* individuals. Results for the *BEAST analysis were obtained by combining log and tree files from three separate runs of 40 million generations each with samples retained from the log and tree files every 1000$^{th}$ generation. Convergence statistics were monitored as described above.

# Results and Discussion

## Field data

We collected 48 specimens (mean snout-to-vent length [SVL] = 864.5 mm ± 225.9, range SVL = 477.0 – 1510.0 mm) in December 2009 and 16 in April 2011 (mean SVL = 870.3 mm ± 138.6, range SVL = 638.0 – 1155.0 mm) on Guam. We captured substantially fewer snakes from the two study sites in Papua New Guinea, despite a more intensive sampling effort in terms of total numbers of days and hours spent searching. This was expected given the abnormally high density of snakes on Guam and the greater complexity of the physical environment in the native range (taller trees, increased floral diversity, etc.). Only four specimens (mean SVL = 772.3 ± 50.5, range SVL = 712.0 – 821.0) were collected from the Kamiali Wildlife Reserve on the north coast of Papua New Guinea over a two-week period (May 2010). Previous surveys conducted by professional colleagues indicated that this was unusual for *B. irregularis* at this site and may have been related to the unusually dry conditions experienced during our sampling period (A. Allison, Bishop Museum, Honolulu, Hawaii; B. Iova, National Museum, Port Moresby, Papua New Guinea: pers. comm.). *Boiga irregularis* appears to be most active after rainfall events at night; indeed, the four specimens we collected in Kamiali were found during or immediately following downpours. *Boiga* density was notably higher on the Admiralty Islands (Manus, Los Negros and Pityilu Islands), where 12 specimens (mean SVL = 1100.7 ± 249.5, range SVL = 573.0 – 1548.0) were collected over a six-day sampling period.

We also collected representatives from six additional snake species for parasite analyses. In Papua New Guinea, these included the Death Adder *Acanthophis laevis* (*n* = 1), the Pacific Ground Boa *Candoia paulsoni* (4), the Slatey-grey Snake *Stegonotus cucullatus* (4), the Northern Tree Snake *Dendrelaphis calligaster* (3), and the Small-eyed snake *Micropechis ikaheka* (1). Although *M. ikaheka* was predicted to occur at Kamiali, this was the first capture record for this species at this site. On Guam, we also captured three small adult blind snakes (*R. braminus*).

## Genetic diversity

Levels of sequence divergence varied substantially among the different loci, with the mitochondrial ND4 gene having the greatest number of informative substitutions (Table 1). The nuclear genes varied as follows (in decreasing order for number of informative characters); *Bi*TBP, MyHC-2, RAG-1 and *Cmos.* We detected heterozygous individuals for all nuclear loci for both length and site-specific polymorphisms, with MyHC-2 showing the highest heterozygosity within individuals. All length variants were unambiguously resolved, whereas some of the site-specific polymorphisms could not be determined with *PP*s > 0.90. Length variants were most common in the *Bi*TBP gene, with a few deletions being parsimony informative within *B. irregularis*. Nearly every *B. irregularis* in the dataset had a 300 base pair insertion within the *Bi*TBP intron that none of the outgroup taxa possessed – only one individual from Indonesia (Pulau Tahulandang, Sulawesi) lacked this insertion and retained the ancestral gene length recovered in all outgroup taxa. Several snakes from the D'Entrecasteaux Islands (Fergusson and Normanby Islands) also had a synapomorphic deletion in the *Bi*TBP gene not found in any other *B. irregularis*. Site-

specific ambiguities that could not be resolved with a posterior >0.90 were conservatively scored as missing data. This mainly affected the outgroup species, as the accuracy of phase inference is expected to decrease as the amount of divergence among terminal taxa grows larger. Even with this conservative approach, we still gained considerable phylogenetic information by teasing out the different allelic phases within individuals (Table 2).

**Table 1.** Summary data for the various genetic markers used in this study. Data are shown for all taxa (outgroup species & *B. irregularis*) and for *B. irregularis* only. Column headers are as follows: No. Characters = number of total nucleotide sites (nt) in the gene sequence; VPU = variable parsimony informative characters; PI = parsimony informative characters. 'Indel' refers to nucleotide insertions or deletions.

| **Marker** | **No. Characters** | **All taxa** | **-** | ***B. irregularis*** | |
|---|---|---|---|---|---|
| | (nt) | VPU | PI | VPU | PI |
| ND4 + tRNAs | 1561 | 189 | 457 | 89 | 202 |
| RAG-1 | 1054 | 44 | 38 | 9 | 10 |
| *C*mos | 954 | 6 | 47 | 4 | 7 |
| My-HC2 | 561 | 12 | 85 | 6 | 14 |
| My-HC2 indel | 6 | 0 | 6 | 0 | 0 |
| *Bi*-TBP | 1014 | 25 | 94 | 17 | 26 |
| *Bi*-TBP indel | 21 | 6 | 15 | 2 | 3 |

No mtDNA variation exists in *B. irregularis* on Guam – we recovered a single haplotype for all 24 snakes sequenced from eight locations across the island, suggesting that the Guam population was founded by an extremely small number of snakes from a single source. In contrast to the complete lack of mtDNA variation, we detected some allelic differences for each of the nuclear genes, although the differences involved single-site substitutions and no more than two alleles per locus were recovered from all of the Guam snakes combined. These allelic polymorphisms reflect the retention of ancestral variation from the source population rather than *in situ* evolution on Guam, given that 'Guam alleles' were detected in the Admiralties for all nuclear genes. In fact, incomplete lineage sorting in the nuclear genes is a general pattern across all populations in the dataset – this was demonstrated by the relatively high nuclear diversity overall, but with few shared derived characters for diagnosing regional groups.

Similar to Guam, there was remarkably little mtDNA diversity on Manus and Los Negros. In fact, we detected only two unique haplotypes among our 12 samples, with only three segregating sites between the two haplotypes. One was recovered at a markedly higher frequency than the other (0.85), although both haplotypes were found on Manus and Los Negros. Nuclear diversity was elevated compared to mtDNA diversity, but again, we attribute this to the general tendency of these snakes to retain ancestral variation. Thus, from the perspective of mtDNA, the Guam population was founded from an already genetically depauperate source, which is not entirely surprising given that Manus/Los Negros is a relatively small island (2100 $km^2$).

Mainland Papua New Guinea and Indonesian populations were notably more diverse at all loci, likely because of their increased opportunity for isolation and divergence over larger land areas. Because most of the mainland New Guinea and Indonesia sampling

sites were represented by single individuals, we pooled subsets of samples according to region and phylogeography to calculate the most meaningful estimates of diversity (Table 2). These groups include 'Kamiali' (collection sites in the vicinity of the Kamiali Wildlife Preserve in the Morobe Province), 'Halmahera' (Indonesian sites exclusive to Halmahera), 'eastern Papua New Guinea' (Oro and Central Provinces, including Cape Nelson), 'North Coast' (populations north of the Huon Peninsula, including KarKar Island), 'Milne Bay' (D'Entrecasteaux Islands), 'Guam' (all locations on Guam), and the Admiralties (Manus, Los Negros, and Pityilu Islands). The 'eastern Papua New Guinea' group has the highest allelic diversity for most nuclear genes, and six distinctive mtDNA haplotypes were recovered from seven individuals – this was not surprising given that the populations within this group are spread over a wide area and occur at higher elevations (i.e. diversity may be elevated as an artifact of how we grouped samples). The most relevant comparisons to Guam are Halmahera and the Admiralties; Halmahera consistently ranked among the highest in diversity estimates for all genes, with the Admiralties generally ranking lower (although not always, depending in the index). Of the island groups, Halmahera was expected to be higher than most others given its larger land area and because of the older age of its *B. irregularis* populations (see next section).

**Table 2**. Summary of genetic diversity indices by locus. Column headers are as follows: No. haplotypes/alleles = number of haplotypes (mtDNA) or alleles (nuDNA); π = nucleotide diversity; $H_d$ = haplotype (mtDNA) and allelic (nuDNA) diversity; *S* = number of segregating sites; *k* = average number of differences in pairwise comparisons among haplotypes. Sites consist of data pooled from several individuals as described in the text on page 18.

| **MtDNA** | | | | | |
|---|---|---|---|---|---|
| **Site** | **No. Haplotypes** | π | $H_d$ | ***S*** | ***k*** |
| Morobe (Kamiali) | 4 | 0.0034 | 0.810 | 9 | 4.29 |
| Halmahera | 5 | 0.0201 | 1.000 | 41 | 25.50 |
| Eastern PNG | 6 | 0.0315 | 0.952 | 73 | 34.19 |
| North Coast | 3 | 0.0100 | 0.833 | 23 | 11.83 |
| Milne Bay | 4 | 0.0381 | 1.000 | 50 | 25.33 |
| Guam | 1 | 0.0000 | 0.000 | 0 | 0.00 |
| Admiralties | 2 | 0.0007 | 0.282 | 3 | 0.85 |

| **RAG-1** | | | | | |
|---|---|---|---|---|---|
| **Site** | **No. Alleles** | π | $H_d$ | ***S*** | ***k*** |
| Morobe (Kamiali) | 4 | 0.0017 | 0.692 | 5 | 1.82 |
| Halmahera | 4 | 0.0011 | 0.533 | 6 | 1.20 |
| Eastern PNG | 8 | 0.0044 | 0.901 | 11 | 4.64 |
| North Coast | 3 | 0.0010 | 0.378 | 3 | 0.60 |
| Milne Bay | 2 | 0.0040 | 0.429 | 1 | 0.43 |
| Guam | 2 | 0.0030 | 0.303 | 1 | 0.30 |
| Admiralties | 8 | 0.0090 | 0.695 | 5 | 1.04 |

| **MyHC-2** | | | | | |
|---|---|---|---|---|---|
| **Site** | **No. Alleles** | π | $H_d$ | ***S*** | ***k*** |
| Morobe (Kamiali) | 4 | 0.0014 | 0.495 | 4 | 0.79 |
| Halmahera | 10 | 0.0083 | 1.000 | 12 | 4.60 |
| Eastern PNG | 4 | 0.0030 | 0.714 | 4 | 1.68 |
| North Coast | 1 | 0.0000 | 0.000 | 0 | 0.00 |
| Milne Bay | 3 | 0.0009 | 0.464 | 2 | 0.50 |
| Guam | 4 | 0.0013 | 0.561 | 2 | 0.71 |
| Admiralties | 4 | 0.0014 | 0.545 | 3 | 0.79 |

| ***C*mos** | | | | | |
|---|---|---|---|---|---|
| **Site** | **No. Alleles** | π | $H_d$ | ***S*** | ***k*** |
| Morobe (Kamiali) | 1 | 0.0000 | 0.000 | 0 | 0.00 |
| Halmahera | 3 | 0.0008 | 0.600 | 2 | 0.73 |
| Eastern PNG | 8 | 0.0018 | 0.901 | 6 | 1.67 |
| North Coast | 1 | 0.0000 | 0.000 | 0 | 0.00 |
| Milne Bay | 2 | 0.0005 | 0.429 | 1 | 0.43 |
| Guam | 2 | 0.0004 | 0.409 | 1 | 0.41 |
| Admiralties | 3 | 0.0004 | 0.394 | 2 | 0.42 |

| **BiTBP** | | | | | |
|---|---|---|---|---|---|
| **Site** | **No. Alleles** | π | $H_d$ | ***S*** | ***k*** |
| Morobe (Kamiali) | 6 | 0.0014 | 0.791 | 4 | 1.40 |
| Halmahera | 5 | 0.0024 | 0.667 | 9 | 2.22 |
| Eastern PNG | 8 | 0.0020 | 0.868 | 9 | 1.97 |
| North Coast | 4 | 0.0017 | 0.750 | 4 | 1.68 |
| Milne Bay | 4 | 0.0022 | 0.857 | 5 | 2.14 |
| Guam | 3 | 0.0007 | 0.689 | 2 | 0.91 |
| Admiralties | 9 | 0.0014 | 0.790 | 10 | 1.42 |

**Phylogeographic inference**

There was general agreement in the inferred tree topologies when comparing our results based on the different combinations of datasets and analyses. The mtDNA gene tree had the highest number of strongly supported branches, as expected given the more rapid rates of mtDNA evolution and greater number of informative substitutions compared to the nuclear genes (Table 2). Not surprisingly, the nuclear genes provided limited resolution toward the tree tips, but did provide some useful information for a few of the deeper nodes in the tree. This pattern was mainly caused by the retention of ancestral allelic polymorphism across populations, such that treesnakes from different localities often shared the same alleles.

Nonetheless, the overall congruence in the phylogenetic signal among the different genes and the presence of incomplete sorting in the nuclear genes indicated that the *BEAST approach was the most appropriate method for extracting information from all genes in a single analysis. We therefore limit our description of the results mainly to the mtDNA and *BEAST trees given that they represent the most clear-cut summaries of the data.

Tree searches based on mtDNA and *BEAST provided strong statistical support (i.e. *PP*s > 0.90) for a close relationship between *B. irregularis* on Guam and those from the Admiralty Islands – in the mtDNA topology, haplotypes from Guam and the Admiralties formed monophyletic group with a comb-like structure, an expected outcome for individuals with high sequence similarity and for which all members of the group share the same unique substitutions relative to others in the dataset (Fig. 3A). In fact, we detected only one fixed difference between the ND4 sequences on Guam and the Admiralties, and surprisingly none of the treesnakes from either Manus or Los Negros carried the Guam ND4 haplotype. In the multi-gene *BEAST analysis, we again recovered strong support for a Guam-Admiralties sister relationship (Fig. 3B). This result provides unambiguous genetic evidence of a single source population for the Guam invasives and is consistent with earlier work based on scale counts and military transportation records (Rodda et al. 1992, Whittier et al. 2000).

Although a comprehensive study on the historical biogeography of *B. irregularis* was beyond the scope of this work, several phylogeographic patterns are noteworthy and have implications for future parasite prospecting. Most underscore the fact that a simple stepping stone model of island dispersal fails to explain the contemporary distribution of lineages within *B. irregularis*, and that the geographic proximity of populations is not always a good predictor of relatedness. This discordance between genes and geography can be attributed to the considerable movement of tectonic plates and island arc systems in the southwest Pacific that began as far back as the Cenzoic, at a time when the Australian plate separated from Antarctica and began pushing northward (ca. 55MA; Allison 1996; Hall 2002). This displacement, followed by subsequent uplift, volcanism and accretion of myriad terrains along the north coast of Papua New Guinea, along with the southerly movement of the Caroline Plate from the north and the westerly movement of the South Bismarck plate from the east, has influenced the divergence among *B. irregularis* populations both on and off the coast of Papua New Guinea. Below, we mention a few findings that relate to the phylogeography of the species as a whole, but avoid detailed descriptions of possible underlying causes for some of the patterns until sampling gaps can be further filled in (*continued on page 23*):

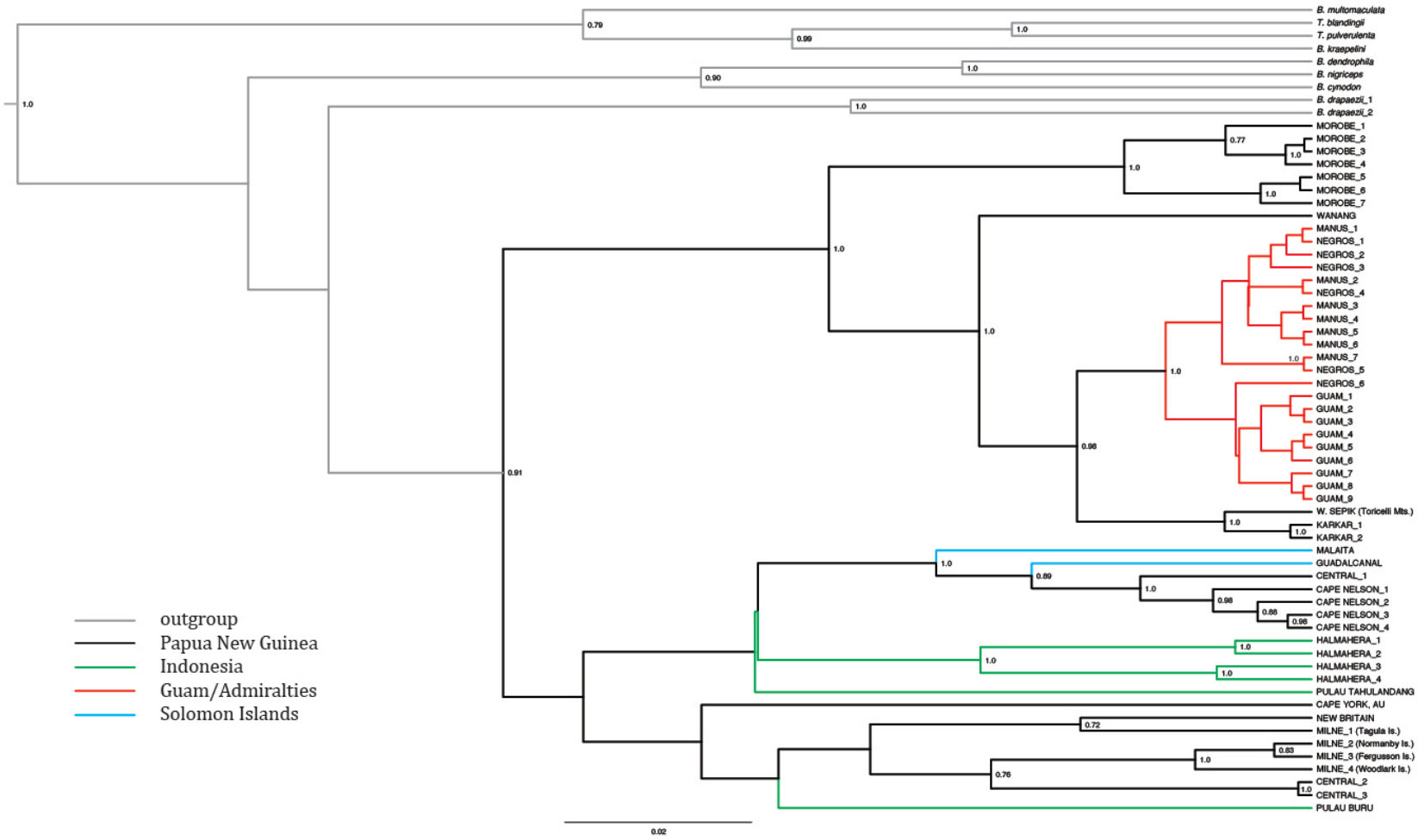



**Figure 3A**. Phylogenetic tree based on mitochondrial ND4 haplotypes. Numbers at the tree nodes are posterior probabilities (only values < 0.70 are shown). Different colored branches indicate different geographic regions.

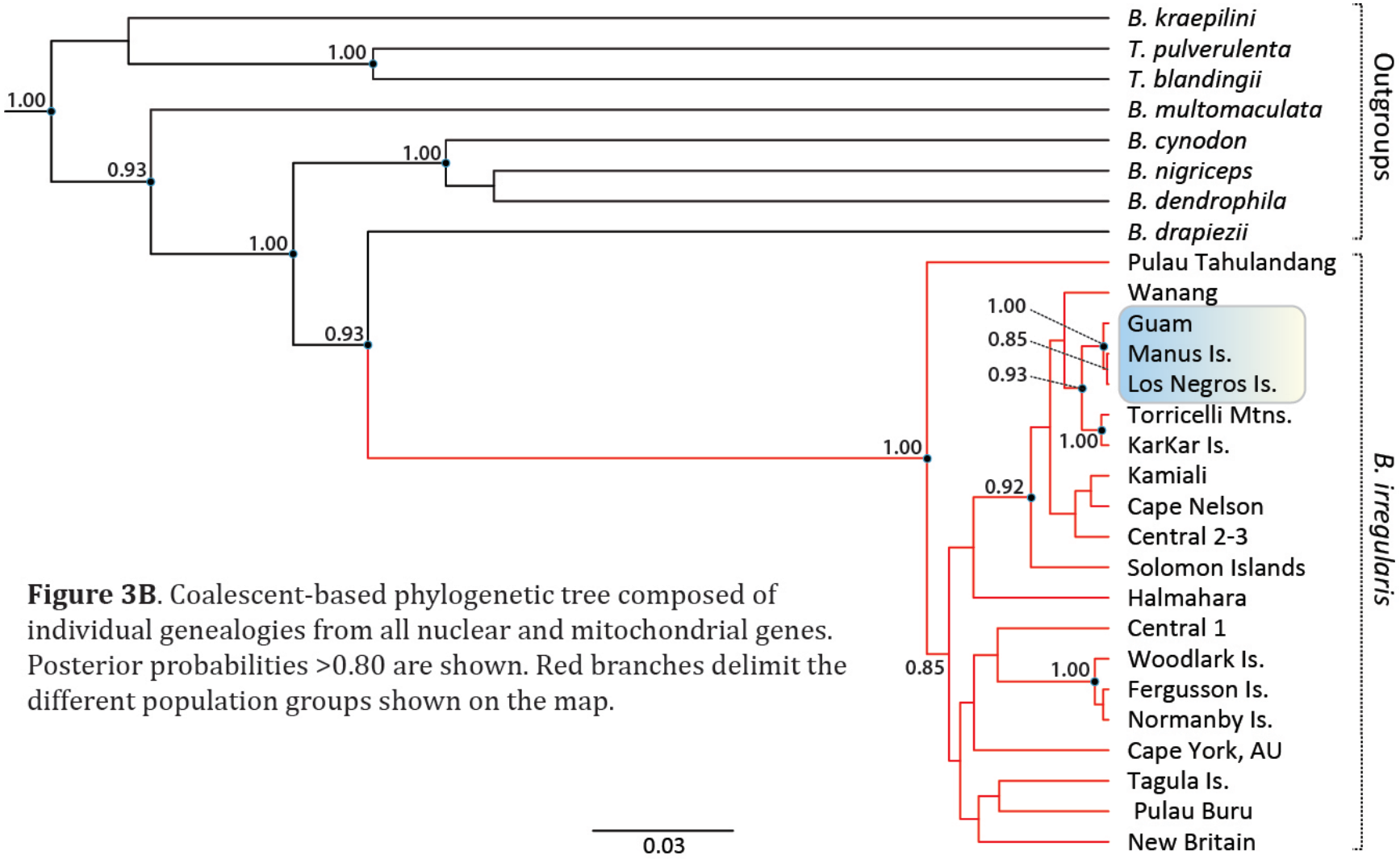



**Figure 3B**. Coalescent-based phylogenetic tree composed of individual genealogies from all nuclear and mitochondrial genes. Posterior probabilities >0.80 are shown. Red branches delimit the different population groups shown on the map.

1. We recovered a close relationship between the Guam/Admiralties group and *B. irregularis* from the Torricelli Mountains on the north coast of Papua New Guinea and on KarKar Island, just east of the city of Madang on mainland Papua New Guinea (Fig 2). Another individual collected in the village of Wanang, also near Madang but located inland, is placed within this group. Although we predicted that *B. irregularis* from the Admiralties would be most closely related to populations in Papua New Guinea (indeed, 81% of the herpetofauna in the Admiralty Archipelago is shared with Papua New Guinea; Allison 1996) the dispersal route by which *B. irregularis* got to the Admiralties was unclear. Two general pathways were plausible: (1) island hopping via historical placement and configurations of the different land fragments now forming the Bismarck (New Hanover, New Britain, and New Ireland) and Admiralty Archipelagos, or (2) by direct overwater dispersal from the north coast of mainland Papua New Guinea.

   Our current data do little to resolve the issue. The once closer proximity of fragments now forming the Bismarcks and the Admiralties suggests that treesnakes could have more easily dispersed between the two island chains in the past – Manus and New Britain are estimated to have been less than 50 km apart approximately 3.5 MA (Taylor 1979, Allison 1996, Hall 2002), which would have increased the probability of successful overwater dispersal. Once on New Britain, the westward displacement of the South Bismarck Plate could have facilitated the translocation of snake populations to the north coast of Papua New Guinea along with land elements that now form the Huon Peninsula[1] (Allison 1996). Of course, we cannot rule out the possibility of direct, open-ocean dispersal from mainland New Guinea to the Admiralties, as there are numerous, well-documented examples of reptiles successfully dispersing over large expanses of ocean (e.g. Fiji iguanas (Cogger 1974, Gibbons 1985), Galápagos iguanas (Rassmann 1997), and Caribbean Iguanas (Malone et al. 2000)). Further molecular data collection on New Hanover, New Britain, and New Ireland populations should help to resolve this intriguing issue.

2. *Boiga irregularis* occurring north of the Huon Peninsula on Papua New Guinea have greater evolutionary affinities to populations in the Admiralty Archipelago than those south of the Peninsula – the collision of the south Bismarck plate (which includes the present-day Huon Peninsula and the Bismarck Archipelago) with the north coast of New Guinea approximately 5-4MA followed by the uplift of the mountain ranges now forming the Huon Peninsula in the late Pliocene (Chappell 1974), may have acted as a dispersal barrier that maintained the genetic separation of *B. irregularis* on the north and south sides of the Peninsula. Indeed, montane areas of the Huon Peninsula (much of which is above 2000 m) harbor very few species of frogs, lizards and snakes, almost none of which are endemics (Allison 1996).

3. Some populations from the eastern tip of Papua New Guinea and the Solomon Islands show closer evolutionary affinities with Indonesian *B. irregularis* than to other

[1] In fact, the Huon Peninsula itself is a remnant of the South Bismarck Plate, and its collision with the north coast of Papua New Guinea is responsible for the uplift of the Adelbert, Finisterre, and Saruwaged Mountains.

populations in Papua New Guinea, even though mainland New Guinea is geographically poised between those two areas. Although the precise relationships of these populations could not be fully resolved, our findings suggest that *B. irregularis* did not use mainland Papua New Guinea as a conduit to reach the southern-most locations of the current range. Rather, these patterns are indicative of ancient dispersal pathways created by island arc systems that were active during the Tertiary, prior to the northward displacement of Papua New Guinea in the mid-Miocene (ca. 10MA or later). Their placement and orientation would have provided land bridges or substantially shorter over-water dispersal distances that allowed at least a few Southeast Asian groups to bypass New Guinea altogether and reach areas further to the east (Allison 1996)

4. We recovered strong statistical support for a sister group relationship between *B. drapiezii* and *B. irregularis*; the geographic range of the former does not extend as far south or east as Papua New Guinea (our samples were from Thailand), but the two species do overlap in parts of the Indo-Pacific Basin. A single *B. irregularis* from Pulau Tahulandang in north Sulawesi is sister to all remaining *B. irregularis* in our dataset – this result, combined with the sister group relationship between the *B. drapiezii* and *B. irregularis*, suggests that the two species likely diverged from a common ancestor in the Indo-Pacific region, followed by dispersal of *B. irregularis* to more southerly locations.

One of our main objectives was to generate a phylogenetic tree that could serve as an evolutionary guide for future parasite and pathogen prospecting. It is clear that the initial parasite surveys constituting this work were performed on the closest relatives to the invasives on Guam (i.e. Admiralties), and although further work is needed in these populations, particularly on viral, bacterial and protozoan pathogens, we now have substantial data collected for a phylogenetically close relative. Ideally, the next surveys would also target populations with intermediate and large phylogenetic distances from the Guam population. The current data identifies several well-supported evolutionary lineages with varying levels of relatedness – treesnake populations from the Morobe Province could fulfill the 'intermediate' category, whereas populations on the D'Entrecasteaux Islands (Goodenough, Fergusson, and Normanby Islands) or Halmahera could represent the 'distant' category.

Substantial progress has already been made for Morobe populations, and the experience and logistic connections gained from this work should enable us to move quickly in gathering more data. In addition to satisfying the 'distant relative' category, populations from Halmahera and the D'Entrecasteaux Islands are appealing for study because they both form well-supported monophyletic groups, and substantial genetic variation exists over relatively small areas. By targeting these locations, we may increase our potential to recover a wide breadth of parasites and pathogens without the need to travel large distances, given that the genetically diverse *B. irregularis* populations in these areas may also harbor taxonomically diverse parasites not yet encountered at our study sites thus far. Also, working with monophyletic groups could prove advantageous in future experimental studies by acting as a type of 'control', or for minimizing potentially confounding covariates in statistical analyses, especially if certain pathogens or parasites

are found to be clade specific.

**Helminth parasites, Guam**

Twenty-four percent of all treesnakes surveyed for parasites on Guam (*n* = 15/62) were positive for helminth infections (Table 3). For both sampling years, the most prevalent helminthes were cestodes referable to the genus *Oochoristica*, accounting for 70% of the infections. Nematodes were the second most prevalent, accounting for 25% of the infections, while trematodes accounted for only 15% of the infections. Only two of the Guam snakes were positive for more than one species of worm, with no more than two species detected per snake.

All of the helminths recovered from our necropsies on Guam snakes have low host-specificity and none are known to be lethal to the host (although some, such as *Kalicephalus*, affect condition). The trematode species *Allopharynx macallisteri* and *Paradistomum mutabile* were likely acquired from the diet, as lizards are the typical hosts. In fact, at the time of necropsy, the single treesnake specimen from which we harvested *A. macallisteri* had a partially digested house gecko (*Hemidactylus frenatus*) in the gut, a known host for *A. macallisteri* (Goldberg et al. 1998). Strongylid nematodes of the genus *Kalicephalus* commonly infect the intestines of lizards and snakes – approximately 23 species have been reported from many regions of the world (Schad 1962, 1964). Route of infection specifically for *Kalicephalus viperae chunkingensis* is not known, but Schad (1956) suggested that larvae of these species might be ingested when snakes use chemosensory tongue flicking to test the environment (Schad 1956). *Kalicephalus viperae chunkingensis* is also known to infect the blind snake *R. braminus* in the Philippine Islands, but we did not detect any *Kalicephalus* in the three *R. braminus* specimens examined from Guam. Approximately 80 species have been described in the cosmopolitan genus *Oochoristica* (Bursey and Goldberg 1996a, b, Bursey et al. 1996, Bursey et al. 1997, Brooks et al. 1999). These anoplocephalid cestodes predominantly parasitize lizards, but some are known to infect snakes, turtles and a few marsupials (Schmidt 1986, Beveridge 1994).

Subsequent to the field collections for this study, colleagues examining treesnake diet on Guam discovered an additional helminth that was never detected in our own surveys. From three snakes, plerocercoid larvae matching descriptions of the cestode *Ligula intestinalis* were recovered from two locations on the island. This is an unusual finding given that snakes are not a known host for this worm. Typical hosts at different stages of the worm's life cycle include copepods (initial host), cyprinid fish (intermediate host), and ultimately a piscivorous avian predator, the latter two of which are rare on Guam. If further study confirms the identity of these species, this finding would suggest a dramatic host shift in the life cycle of this worm, possibly instigated by a lack of the more typical cyprinid and avian hosts. Of further interest is that fish hosts infected with *L. intestinalis* plerocercoids are unable to reproduce – their gonads fail to develop and there is (*continued on page 27*)

**Table 3**. Parasite identity, site of infection, prevalence (proportion of infected hosts among all hosts examined), and mean intensity (number of helminths per infected individual). Values are distinguished for Guam and Papua New Guinea (PNG). Light blue rows indicate helminths that were detected in snake species other than *Boiga* in Papua New Guinea. Note that some snakes were parasitized by more than one helminth species.

| Helminth species | Site of Infection | Prevalence (Guam) Number | (%) | Intensity (Guam) Mean ± SD | Prevalence (PNG) Number | (%) | Intensity (PNG) Mean ± SD |
|---|---|---|---|---|---|---|---|
| **Trematoda** (flukes) | | | | | | | |
| *Allopharynx macallisteri* [1] | Intestine | 1 | 1.6 | 2 | 0 | 0.0 | 0 |
| *Paradistomum mutabile* [1] | Intestine | 1 | 1.6 | 3 | 0 | 0.0 | 0 |
| Unidentified trema | Intestine | 0 | 0.0 | 0 | 5 | 31.3 | 125.0 ± 129.3 |
| **Cestoda** (tapeworms) | | | | | | | |
| *Oochoristica* sp. | Intestine | 9 | 14.5 | 2.6 ± 2.8 | 2 | 12.5 | 1.5 ± 0.7 |
| *Ligula intestinalis*[2] (plerocercoid larvae) | Subcutaneous/Coelom | ? | ? | ? ? | 0 | 0.0 | 0 |
| **Nematoda** (round worms) | | | | | | | |
| *Kalicephalus viperae chunkingensis* | Intestine | 3 | 4.8 | 3.0 ± 2.6 | 0 | 0.0 | 0 |
| *Kalicephalus novae-brittanniae* | Trachea | 0 | 0.0 | 0 | 2 | 12.5 | 2.0 ± 1.4 |
| *Kalicephalus costatus indicus* | Intestine | 0 | 0.0 | 0 | 1 | 6.2 | 1 |
| *Kalicephalus posterovulvus* | | – | – | – – | 1 | 7.7 | 2 |
| *Meteterakis* sp. | Intestine | 1 | 1.6 | 1 | 0 | 0.0 | 0 |
| *Ascarididae* sp. | Intestine | 0 | 0.0 | 0 | 1 | 6.2 | 1 |
| *Abbreviata* sp. | Stomach | – | – | – – | 2 | 15.4 | 5.0 ± 1.4 |
| **Pentastomida** (tongue worms) | | | | | | | |
| *Kiricephalus tortus* | Lung/Coelom/Intestine | 0 | 0.0 | 0 | 12 | 75.0 | 5.8 ± 7.1 |
| *Parasambonia bridgesi* | Lung/Coelom | – | – | – – | 2 | 15.4 | 1.0 ± 0.0 |
| *Waddycephalus punctulatus* | Lung/Coelom | – | – | – – | 2 | 15.4 | 7.0 ± 8.5 |

[1] Likely acquired from the diet, as lizards are the typical hosts.

[2] Morphological characters are consistent with *L. intestinalis*; however, we consider this a tentative identification because snakes are not a known host for this worm.

an apparent suppression of the presumed gonadotropin producing cells in their pituitary glands (Arme et al. 1982, Carter et al. 2005). The exact mechanism by which this occurs is unclear, but if treesnakes are fulfilling the role of an intermediate or definitive host on Guam, the effects of infection on reproductive success would be worthy of investigation.

Most importantly with respect to this study, one of the snakes infected with *L. intestinalis* showed obvious signs of pathology (J. Stanford, USGS; pers. obs.) – the body cavity was inundated with larvae, many of which were protruding through the body wall and causing necrosis at the site of penetration. Numerous larvae were also encapsulated in pustules beneath the skin, and the snake was conspicuously lethargic while being handled. We note that this particular individual had an unusually heavy infection load relative to others harboring these same worms, and that none of the other snakes displayed the same obvious signs of pathology (E. Wostl; CSG Dynamic, pers. comm.). This could reflect differences in the progression of the life cycle of the worm with respect to the timing of the snake's capture, or that individuals differ in how they respond to infection. Further study on prevalence, hosts, and a confirmed taxonomic identity based on molecular data are needed for this species. Since we received the initial specimens from our colleagues on Guam, more snakes have been encountered with these same larvae, and most were recovered from a single location (E. Wostl; CSG Dynamic, pers. comm.). Because of this worm's apparent ability to induce pathology in treesnakes, at least in some individuals, additional field efforts targeting this species are warranted.

In conclusion, the low-prevalence of infected snakes and the limited taxonomic diversity of the helminths that infect them suggest that at least some of the ecological success of *B. irregularis* on Guam may be attributable to the lack of helminths parasites. Performance and fitness tests are necessary to definitively test this hypothesis, but when compared to *B. irregularis* surveyed in Papua New Guinea (see next section), far fewer infected snakes were recovered on Guam and their infection loads were notably lower. This general lack of macroparasites bodes well for the prospects of biological control, given that the immune response of Guam snakes may be dampened as a consequence of the lack of persistent, parasitic challenges that are normally experienced in the native range.

**Helminth parasites, Papua New Guinea**

In contrast to Guam, nearly all of the *B. irregularis* captured in Papua New Guinea were positive for helminth parasites ($n$ = 14/16, or 88%; Table 3). The most prevalent were pentastomid tongue worms in the genus *Kiricephalus* – 75% of the treesnakes ($n$ = 12/16) infected with helminths were positive for *Kiricephalus tortus*, a species that was specific to *B. irregularis* (Fig. 4). Other pentastomids included *Parasambonia bridgesi*, which was specific to the Ground Boa *C. paulsoni*, and *Waddycephalus punctulatus*, which was detected in both *C. paulsoni* and the Slatey-grey snake *Stegonotus cucullatus.* The second most prevalent worms were unidentified trema species ($n$ = 5/16 infected snakes), followed by nematodes ($n$ = 3/16) and cestodes ($n$ = 2/16). One of the two nematode species detected in *Boiga*, *Kalicephalus novae-brittanniae*, was also recovered from a Dead Adder (*A. laevis*), whereas the other *Kalicephalus* species, *Kalicephalus costatus indicus*, was found only in *B. irregularis.* Seven of the 14 infected *B. irregularis* were positive for two or more helminth

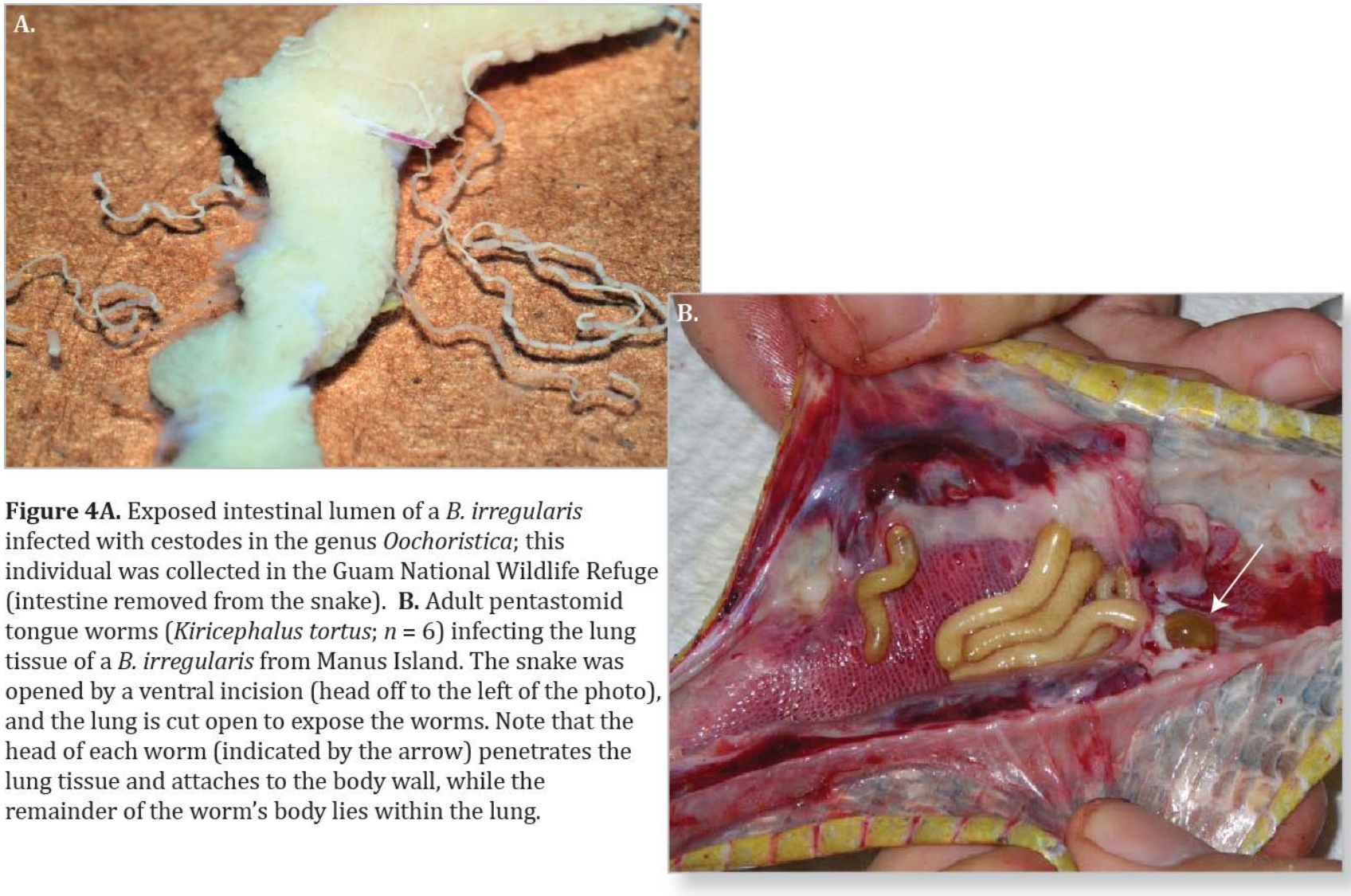



**Figure 4A.** Exposed intestinal lumen of a *B. irregularis* infected with cestodes in the genus *Oochoristica*; this individual was collected in the Guam National Wildlife Refuge (intestine removed from the snake). **B.** Adult pentastomid tongue worms (*Kiricephalus tortus*; $n = 6$) infecting the lung tissue of a *B. irregularis* from Manus Island. The snake was opened by a ventral incision (head off to the left of the photo), and the lung is cut open to expose the worms. Note that the head of each worm (indicated by the arrow) penetrates the lung tissue and attaches to the body wall, while the remainder of the worm's body lies within the lung.

species, with one individual being heavily infected with four different species at the same time.

Nematodes in the genus *Kalicephalus* and pentastomids in the genus *Kiricephalus* are noteworthy considerations for biocontrol. The former are intestinal residents with long-lived infectious stages and are directly transmitted by fecal-oral routes, thereby avoiding some of the issues associated with complex life cycles or the consumption of infected intermediate hosts. *Kalicephalus* species may also cause loss of condition that results in reproductive costs (Dobson and Altizer 2001). Although the latter likely require intermediate hosts to complete the life cycle, adults of the single *Kiricephalus* species detected in treesnakes from Papua New Guinea, *Kiricephalus tortus*, are host-specific to *B. irregularis* based on all studies to date (Fig. 4B: Shipley 1898, Ewers 1973, Riley and Self 1980). These large worms occupy significant lung volume in the adult stage and are generally long-lived (~130 species of extant pentastomids; six recognized species of *Kiricephalus*). Nymphs are also known to stimulate severe inflammatory responses in the host, although little outward pathology has been documented for definitive reptilian hosts (Roberts and Janovy 2005). The life cycle and host progression of *K. tortus* is poorly known, other than the apparent adult host-specificity to *B. irregularis*; nymphs of other *Kiricephalus* species infect diverse taxa, including amphibians, lizards, snakes and mammals (Riley and Self 1980). Because we were able to obtain substantial information about *B. irregularis* diet through gut dissections, it should be possible to identify the range of intermediate hosts for *K. tortus* in future studies.

The main result of these helminth surveys is that none of the species recovered from *B. irregularis* in Papua New Guinea or the Admiralty Islands were found in any snakes sampled on Guam. Only one helminth species recovered from a single individual on Guam was also recovered from a snake in Papua New Guinea – the strongylid nematode *Kalicephalus viperae chunkingensis* was also detected in a small-eyed snake *M. ikaheka* collected at Kamiali, but interestingly not in any of the *B. irregularis* collected from the same area. This confirms the low host specificity for this species, which is typical of *Kalicephalus* in general, and suggests that *K. viperae chunkingensis* would probably be detected in *B. irregularis* with a larger sample size from Kamiali. Nonetheless, the taxonomic discordance of the helminth faunas between the source and introduced population, combined with the low prevalence and diversity of helminths detected in Guam snakes, is consistent with the hypothesis of enemy release (Keane and Crawley 2002, Mitchell and Power 2003, Torchin et al. 2003).

## Hemoparasites

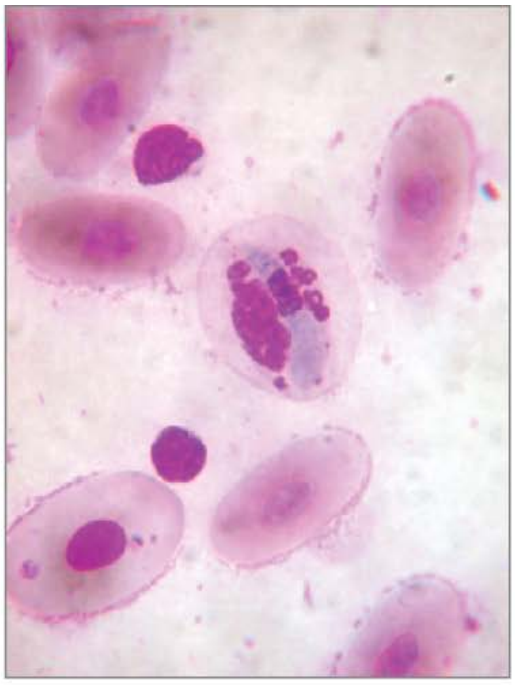
Intraerythrocytic bodies identified as haemogregarine gamonts (i.e. life cycle stage that is committed to undergoing gametogenesis) were found in 75% of 16 brown tree snakes collected in Kamiali and the Admiralty Islands (Fig. 5) – we did not find haemogregarines in any smears prepared from Guam snakes. A similar pattern of high haemogregarine prevalence has also been shown for *B. irregularis* in Queensland and the Northern Territory of Australia (Jake et al. 2003; Caudell et al. 2002), but interestingly not in the Solomon Islands (Jakes et al. 2003). The complete absence of haemogregarines in *B. irregularis* on Guam is consistent with a previous survey conducted in the mid- 1990's (Jakes et al. 2003),

suggesting that the original propagules were either not infected with haemogregarines, or that infections could not be sustained due to a lack of appropriate vectors.

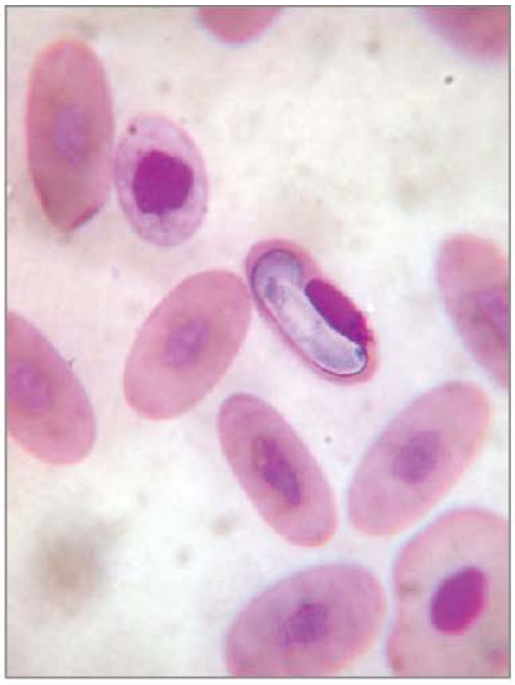
Haemogregarines are protozoans that infect and multiply in host erythrocytes. They are the most common intracellular protozoan parasites in snakes, and are conventionally described on the basis of gamont morphology, host occurrence, and location of sporogonic stages in the definitive invertebrate host (Telford 2009). Effects of these protozoans on host fitness have been debated, with some studies detecting no or negligible effects of infection in lizards, snakes, and birds (Weatherhead 1990, Dale et al. 1996, Wozniak et al. 1996, Eisen 2001, Jakes et al. 2003). In contrast, others have demonstrated severe pathological and life history effects, including leuko- and erythrocytosis, DNA fragmentation, anorexia, myopathy, reduced growth rates and reproductive output, and death (Atkinson and van Riper 1991, Wozniak et al. 1996, Oppliger and Clobert 1997, Merino et al. 2000, Madsen et al. 2005). At least some evidence to date indicates that haemogregarine infections may not cause significant pathology in *B. irregularis* during their schizogonous proliferation in tissues or their gamont formation in erythrocytes (Jakes et al. 2003); however, data on this topic are limited and mainly involve qualitative assessments of condition, rather than quantitative studies comparing gene expression between infected and non-infected snakes. Furthermore, effects on the Guam population may be quite different from treesnakes in the native range that are continuously exposed to these parasites.

Comparison of the developmental stages in our blood smears, combined with the observation that no schizonts (i.e. cells undergoing a form of asexual reproduction in which multiple mitoses take place, giving rise to many daughter cells at once) were detected in erythrocytes, suggests that the haemogregarines in our samples are likely encapsulated gamonts of *Hepatozoon* species awaiting uptake by a suitable vector. *Hepatozoon* is a genus of Ampicomplexan protozoa that includes over 300 species of obligate intraerythrocytic parasites with heteroxenous life cycles – different reproductive stages occur within vertebrate and haematophagous invertebrate hosts. Exotid ticks, Anopheline and culicine mosquitoes, and phlebotimine sand flies are known experimental vectors (Telford Jr. 1984). Individuals typically become infected through bites or ingestion of infected intermediate hosts; however, the life cycles, vectors and host specificity of most haemogregarine species are poorly known, making it difficult to determine whether a lack of suitable vectors explains their absence in *Boiga* on Guam and the Solomon Islands. In either case, it is also possible that a small number of original founders were simply not infected upon arrival.

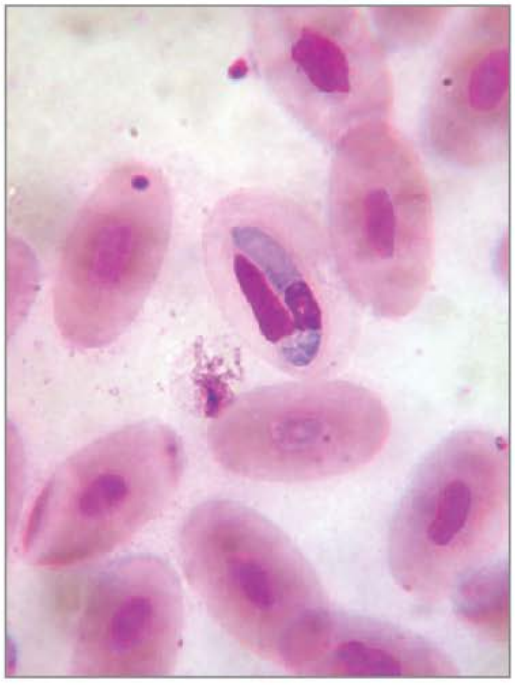
The identification of *Hepatozoon* spp. in snakes has been based primarily on the morphology of gamonts, cysts in the inner organs of vertebrate hosts, and oocytes in vectors. Although we are unable to definitively reconcile the ultrastructure of the putative *Hepatozoon* species within our samples to those in previously published studies, four morphospecies were clearly distinguishable in blood smears collected from *B. irregularis* on mainland Papua New Guinea and the Admiralty Islands (Fig 5.). The gamonts, which occupied approximately half the volume of infected erythrocytes, differed in size, shape, and the positioning of the nuclei within the gamont. None of the four species appeared to be specific to *Boiga,* although two were recovered only from *Boiga* and *Stegonotus*. The most common was *Hepatozoon* sp. 2, which was found in representatives from all six snake



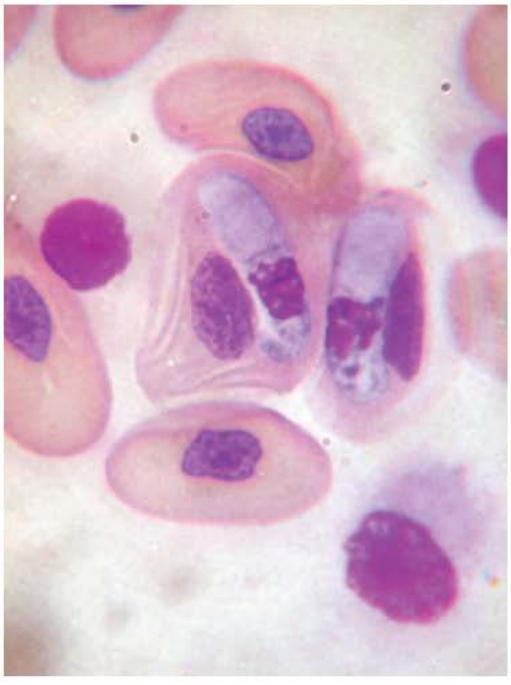
*Hepatozoon* spp. 1



*Hepatozoon* spp. 2



*Hepatozoon* spp. 3



*Hepatozoon* spp. 4

**Figure 5**. Examples of erythrocytes infected by gamonts of the four putative *Hepatozoon* spp. These examples were drawn from different *B. irregularis* individuals in Papua New Guinea.

species (including *B. irregularis*). Of the 12 *B. irregularis* that tested positive for haemogregarines, only one individual was massively infected (captured on Los Negros), possibly with two different *Hepatozoon* species, whereas most had light infections that were barely detectable under light microscopy. At least two of the different gamonts had characters that are consistent with *Hepatozoon boigae*, described from Australian *Boiga* (Mackerras 1961, Jakes et al. 2003), but further confirmation is needed through genetic studies to assess whether our morphospecies correspond to previously diagnosed *Hepatozoon*.

In summary, the most important finding is that the Guam population had no haemogregarines, in contrast to the high prevalence of haemogregarines in the New Guinea samples. This iterates the results from the helminth surveys and supports the hypothesis that *B. irregularis* on Guam have been released of their former, co-evolved parasites, and that at least some of their ecological success is likely due to a lack of natural population controls.

# Conclusions and Implications for Future Research/Implementation

## Overall results and conclusions

This study satisfies the key objectives outlined in our SEED proposal, and provides a critical foundation for guiding further genetic, parasitological, and pathogen-related studies that seek to identify a biological agent(s) for managing invasive *B. irregularis* on Guam. With SEED funding, we were able to make the following conclusions:

1. We definitively identified a genetic source for the Guam *B. irregularis* population, and demonstrated that this population originated from a very small number of snakes from a single location in the Admiralty Islands of Papua New Guinea. Our results also ruled out the possibility of multiple source populations. The identification of a single genetic source is significant because translocations from multiple, separate sources would have resulted in increased genetic diversity on Guam, which could ultimately detract from the efficacy of a biological agent. As this is not the case, we believe that further research on biological management is warranted.

2. We provided a phylogenetic framework that serves as a guide for future parasite prospecting in native populations. With these data, we are able to identify populations that are closely, intermediately and distantly related to the Guam population, providing a broad range of 'evolutionary latitude' among populations from which we can further sample parasites. These data also allowed us to pinpoint places in the native range where substantial genetic variation exists over relatively small areas (e.g. D'Entrecasteaux Islands and Halmahera). By targeting these locations in our follow-up work, we can potentially increase our ability to recover a wide breadth of parasites and pathogens, given that these genetically diverse host populations may also harbor some of the more diverse parasite faunas. We can also continue to build on this already substantial molecular dataset as new treesnake specimens are acquired from different places in the native range, allowing us to fill in knowledge gaps about the historical biogeography of the species.

3. We resolved the longstanding question of whether Guam treesnakes have parasites or not – our results showed that contrary to statements that have persisted in the literature for many years, the Guam snakes do in fact harbor parasites, but those parasites are largely innocuous (with the noted exception of *Ligula intestinalis*) and none appear to have come from the native range. At least two species found in Guam snakes were likely acquired from the diet, as lizards are the typical hosts.

4. We have identified a number of new host records for various helminth parasites, including one potential example of a dramatic host shift in the larval stage of a particular cestode on Guam (this worm induces obvious pathology in at least some snakes). We also identified up to four possible *Hepatozoon* species that infect the erythrocytes of *B. irregularis* in the native range. None of the helminth or haemoprotozoan parasites recovered from New Guinea treesnakes were found in any snakes on Guam. Thus, one of our major conclusions is that the invasive population on

Guam has been released of its native parasites, allowing individuals to persist without the immunological challenges experienced by snakes in the native range. This may explain at least some of their ecological success on Guam and again increases the prospects for successful biological management.

One objective outlined in our SEED proposal was to perform haemoparasite species identifications based on DNA sequence data. We did not specifically meet this objective – instead, at the onset of the study, we realized that a critical first step of the project was to verify in a rigorous, systematic manner whether the Guam snakes harbored any parasites, and if present, whether any of those parasites were derived from native sources. As field trips to Guam were not included as part of the original budget, and per our phone discussion with the SERDP program manager before the study formally began, we reallocated funds from the molecular characterization of the blood parasites and combined with them matching USGS funds to cover the expenses of field studies on Guam. Although we were not able to complete the specific task of molecular parasite identifications at this stage of the project, we currently possess blood samples from all treesnakes collected to date, and from these samples we can extract parasite DNA (some have already been successfully extracted). We also possess the primers necessary to amplify the genes that are used to make these identifications; thus, pending further funding, we will be able to immediately pursue this aspect of the project.

Finally, through the SEED funding we have been able to establish in-country conservation group affiliates (e.g. Wildlife Conservation Society [WCS], Manus Island; Papua New Guinea National Museum) and have assisted with the development of necessary infrastructure on Manus Island to conduct further field work – WCS now has a 4X4 field vehicle, a boat with a large outboard motor for access to nearby satellite islands, a formal office in Lorengau with computers, and a working area for necropsies and specimen preservation. Therefore start-up investment has been largely eliminated, allowing us to more efficiently pursue our follow-up research.

**Potential next steps and objectives for follow-up research**

Despite meeting our SEED objectives, several key questions need to be addressed as we progress further towards identifying candidate biological agents. These questions include the following (bold italics):

***Based on DNA identification, what species of haemogregarine species infect B. irregularis? Are they host-specific to B. irregularis?*** Recently developed molecular tools and a growing DNA sequence database for different parasites makes it possible to test for the presence of haemogregarines using DNA sequence data (Austin and Perkins 2006, Martinsen et al. 2008, Perkins and Austin 2009). These protozoans have been singled-out as some of the most suitable, potential control agents for *B. irregularis* on Guam (Telford Jr. 1999, Caudell et al. 2002), and this study is the first to document their presence in *B. irregularis* from Papua New Guinea. Determining whether these parasites are unrecognized or previously diagnosed *Hepatozoon* spp., identifying routes of infection, and evaluating their host-specificity is a top priority. Because of their documented capacity to induce pathology in snakes, the presence of numerous potential vectors on Guam, and the fact that

Guam snakes have survived for an extended amount of time without infection challenges, haemogregarines show promise as a future biomanagement tool for *B. irregularis*.

***What are the bacterial and viral pathogens that infect native B. irregularis? Besides blood protozoans, what additional protozoans (i.e. amoebas and coccidian) infect the gastrointestinal tracts of B. irregularis?*** These pathogens and parasites require a more rigorous field investigation that was beyond the scope of this first project, and will involve more complex field and lab techniques to isolate, grow and identify, although new DNA techniques are making them easier to diagnose (Landolfi et al. 2010). Assessing risk and population impacts will be part of the investigation. Amebiasis is one of the most significant parasite afflictions of captive snakes and is highly contagious. These organisms cause extensive damage to the intestinal lining and liver, and secondary bacterial infections are common and contribute significantly to the severity of the disease.

***How common is Ligula intestinalis on Guam, and what are the other intermediate hosts? How does this cestode affect treesnake condition?*** Given that *L. intestinalis* has been detected in several snakes on Guam and has been shown to cause pathology in at least some individuals, further investigation on the life cycle, prevalence, effects on treesnake condition and the reasons underlying its apparently restricted distribution on Guam are necessary. DNA sequence data are also needed to verify the taxonomic identity of this worm, given that snakes are not a typical host for this species.

Genetic diversity is often used as a proxy for inferring how well a population might respond to novel pathogens, with lower diversity potentially translating to a reduced ability to fight disease. ***How genetically depauperate is the Guam population relative to the source population? Does the Guam population have limited variation in genes that control the upstream regulation of acquired immunity?*** These questions can be addressed through comparative studies of neutral genetic markers and markers evolving under natural selection, such as major histocompatibility genes, which have implications for understanding disease resistance and susceptibility. The USGS San Diego Field Station recently acquired a next-generation 454 pyrosequencer that is well-suited to identifying genes that 'turn on' once an individual becomes exposed to a pathogen or parasite, providing a rigorous examination of how certain pathogens (e.g. *Hepatozoon* spp.) challenge the immune system and increase stress. We have also recently developed a microsatellite library for Manus Island *B. irregularis* that contains over 1600 neutral microsatellite markers; thus, we are uniquely poised to begin work that can directly compare the immunocompetence of *B. irregularis* on Guam with those in the native range using some of the most modern DNA sequencing equipment and techniques.

***Do Guam treesnakes constitute one panmictic population of randomly mating individuals, or is there detectable population structuring on the island?*** A critical knowledge gap exists regarding certain aspects of treesnake demography on Guam. In particular, nothing is known about the effective population size, the degree of spatial genetic structuring across different parts the island, or the amount gene flow among these areas. As demonstrated in other host-parasite systems, population structuring is predicted to influence the rate of pathogen spread (Shigesada and Kawasaki 1997), the probability of pathogen persistence in the population (Grenfell and Harwood 1997), and decisions regarding the best approach for releasing a biological agent (Shea and Possingham 2000).

By developing 20-30 loci from our existing brown treesnake microsatellite library and using the tissue samples already in hand, we will be able to fill in these critical knowledge gaps, which must be addressed before implementing any form of biological management.

***What factors serve as natural population controls in the native range?*** Treesnakes occur on numerous small islands in the Admiralty Archipelago (most of which are substantially smaller than Guam), yet snake population densities have never exploded as they have on Guam and the islands maintain rich lizard, frog and bird faunas. Predation pressure also appears to be minimal to non-existent. These observations beg the question of why the treesnakes have not exploited these islands in a manner similar to that on Guam, and suggest that parasites and pathogens may be a driving factor in controlling the growth of insular populations in the native range. Understanding the degree to which parasites and pathogens affect population densities would go a long way towards assessing the viability of a successful biological management program on Guam.

Conducting quantitative surveys for several taxon groups on islands spanning three size classes would provide key information on how native *B. irregularis* populations are kept in check under natural conditions. Focal groups would include the same taxa affected by treesnakes on Guam, namely lizards, frogs, and birds. These surveys would be performed in conjunction with parasite and pathogen prospecting on the same islands, and could be done with relatively little additional field effort and cost. One of the targeted islands would include Manus because it is the largest island in the Admiralty Archipelago and we already have preliminary data for some taxa. Accessing two additional islands from Manus, one intermediate and one small, is trivial due its central location in the island chain, and our in-country Wildlife Conservation Society collaborators have recently acquired a power boat that is available for research purposes.

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